

# Biologic Mechanisms for the Regulation of Normal Human Keratinocyte Proliferation and Differentiation

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Normal human keratinocytes can be grown in serum-free medium, and the integrated control of their proliferation and differentiation can be modulated experimentally. The growth of cultured human keratinocytes can also be specifically arrested at either reversible or irreversible growth arrest states. Reversible growth arrest is induced by culture in medium containing TGF- $\beta$  or ethionine or in medium deficient of isoleucine. Irreversible growth arrest is induced by culture in razoxane-containing medium or by routine passage of keratinocytes until senescence results. The current studies were performed to determine from which growth arrest states keratinocyte differentiation occurs. Cells were therefore growth-arrested at each

state, and they were then incubated in several different differentiation-promoting culture conditions. The results show that differentiation, as determined by morphologic, cytochemical, and immunofluorescent assays, can be induced from multiple reversible and irreversible growth arrest states by a series of complex biologic mechanisms. More specifically, at least three distinct stages appear to be involved in the process of keratinocyte differentiation. First, cells arrest their growth at a reversible predifferentiation state. Second, cells irreversibly lose their proliferative potential. Finally, cells express the terminally differentiated keratinocyte phenotype. (Am J Pathol 1988, 131:171-181)

THE EPIDERMIS is an excellent example of a dynamic tissue in which highly regulated mechanisms exist to control cell proliferation and differentiation. The traditional view based primarily on *in vivo* studies in animals is that the proliferative compartment of the epidermis resides in the basal layer and that as basal cells migrate suprabasally, they differentiate and lose their proliferative potential. Nonetheless, the precise biologic mechanisms that regulate the control of proliferation and differentiation in epidermal cells have not been definitively established.

With the ability to culture normal human keratinocytes in serum-free medium and to modulate their proliferation and differentiation experimentally,<sup>1,2</sup> it is now possible to study the mechanisms that influence keratinocyte proliferation and differentiation. In this regard, we previously established that the control of keratinocyte proliferation and differentiation is integrally regulated.<sup>2</sup> That is, if keratinocyte proliferation is promoted, differentiation is inhibited; whereas if differentiation is induced, proliferation is inhibited.

We have also established that the proliferation of human keratinocytes can be regulated by two distinct types of states.<sup>3</sup> One type of growth arrest state is reversible and can be induced by culture in medium containing TGF- $\beta$  or in medium deficient of isoleucine. The other type of growth arrest state is irreversible and can be induced by culture of keratinocytes in medium containing razoxane or by passage in complete medium until senescence results.

In the current studies, we have developed an additional method to reversibly growth arrest keratinocytes by culture in ethionine containing medium, and we have determined what role growth arrest, whether

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reversible or irreversible, plays in the induction of terminal differentiation in normal human keratinocytes. The results show that three specific stages exist in the process of keratinocyte differentiation. First, cells arrest their growth at a reversible predifferentiation state. Second, cells irreversibly lose their proliferative potential. Finally, cells express the terminally differentiated keratinocyte phenotype.

## Materials and Methods

### Cell Culture and Culture Media

The procedures for the isolation and culture of normal human keratinocytes are the same as those previously described.<sup>2</sup> Keratinocyte cultures were routinely grown in complete MCDB 153 medium in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 C. Complete MCDB 153 consists of basal nutrient medium MCDB 153,<sup>4</sup> supplemented with ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), hydrocortisone ( $5 \times 10^{-7}$  M), insulin (5  $\mu$ g/ml), EGF (10 ng/ml), and protein of bovine pituitary extract (BPE) (140  $\mu$ g/ml) with a calcium concentration of 0.1 mM. In some experiments this medium was modified as explained in Results by adding various drugs, by depletion of isoleucine or growth factors, by increasing the calcium concentration to 2 mM, or by deleting BPE from the medium.

### Clonal Growth Assays, Autoradiography, and Cytofluorometry

Clonal growth assays were performed as described.<sup>2,4</sup> Briefly, 500 cells were seeded into 60-mm culture dishes containing complete MCDB 153 medium, and thereafter they were grown for 10 days before fixation, staining, and quantitation of colony formation. Colony-forming efficiency (%CFE) was then determined.<sup>2</sup> Standard procedures for cell counting and autoradiographic analysis were employed.<sup>2</sup> In addition, flow microfluorometry (FMF) was used to characterize the distribution of keratinocytes in either the G<sub>1</sub>, S, or G<sub>2</sub>/M phase of the cell cycle.<sup>2</sup> To distinguish between cells in G<sub>2</sub> or M phase of the cell cycle, morphologic analysis using a Hoechst 33258 stain for DNA was performed. These studies established that most keratinocytes that were growth-arrested with a 4n DNA content were primarily G<sub>2</sub> cell populations, because mitotic cells could not be detected in significant quantity.

### Induction of Reversible and Irreversible Arrest of Keratinocyte Growth

Five specific methods were used to induce the arrest of normal human keratinocyte growth. Most of these

methods were recently described by us<sup>3</sup>; the others are briefly outlined in this paper. Three methods were used to induce initially reversible growth arrest of normal human keratinocytes. These include incubation of cells for 48–72 hours in 1) complete MCDB 153 containing 8 mM ethionine (Aldrich Chemical Company, Milwaukee, Wis), 2) complete MCDB 153 medium containing 5 ng/ml TGF- $\beta$  (R & D Systems, Minneapolis, MN), or 3) complete MCDB 153 that was devoid of isoleucine. Irreversible arrest was induced, for example, by culture of keratinocytes for 48–72 hours in complete MCDB 153 medium containing 20  $\mu$ g/ml razoxane (ICRF 159, Imperial Chemical Industries, PLC, Cheshire, England) or by prolonged culture of keratinocytes in complete MCDB 153 with routine passage at low density ( $<8 \times 10^3$  cells/cm<sup>2</sup>) until senescence occurred, typically after approximately 30–50 population doublings.<sup>2</sup> Arrest of cell growth induced by the above methods was documented by cell count analysis, autoradiography, cytofluorometry, and clonogenic assays.<sup>2</sup>

### Differentiation Induction Procedures

Cultures that had arrested their growth were evaluated for determination of whether they expressed a differentiated phenotype when maintained in the native growth arrest-inducing medium or after being re-fed differentiation-promoting medium and then cultured therein for intervals up to 12 days, at which time differentiation assays were performed again. Such differentiation assays have previously been described in detail elsewhere.<sup>2</sup> The most efficient differentiation-promoting medium consists of complete MCDB 153 containing 2.0 mM Ca<sup>2+</sup>  $\pm$  growth-arresting agent  $\pm$  growth factors.

### Morphologic and Histochemical Assays for Keratinocyte Differentiation

Morphologic differentiation was evaluated by light microscopy for detection of the formation of multilayered differentiated and stratified cellular foci designated “crowns.” Keratinocyte differentiation was also assayed histochemically by use of the method of Ayoub and Shklar,<sup>5,6</sup> wherein differentiated cells stain red and undifferentiated cells stain blue. Quantitation of morphologic and histochemical differentiation assays was based on the percent of cell colonies that displayed a crown or red staining foci, respectively.

### Immunofluorescent Staining With Antiserum to Involucrin

Cultures were dissociated and cell suspensions were then air-dried onto microscope slides and subse-

quently fixed in 3.7% formaldehyde in phosphate buffer, pH 7.4, at room temperature for 8 minutes. Thereafter, they were fixed in methanol (−20 C) for 4 minutes and in acetone (−20 C) for 2 minutes. After three consecutive washes of 5 minutes each with Tris-HCl (0.01 M, pH 7.6)/NaCl (0.15 M) buffer, the slides were stained for involucrin with a commercially obtained staining kit (Biomedical Technologies Inc., Cambridge, Mass). Staining procedures were carried out as described in the kit with the following exception. Slides were incubated with a diluted FITC-conjugated goat anti-rabbit immunoglobulin (Miles Biochemicals, Elkhart, Ind), washed in buffer, and viewed with a Zeiss microscope fitted with ultraviolet illumination and barrier filters to enhance detection of fluorescein fluorescence instead of using the peroxidase method of staining as supplied in the kit. In all the assays described in this paper, cells were scored to show significant involucrin expression only when highly prominent fluorescence was detected that showed a predominant cell surface localization.

#### **Immunofluorescent Staining Using Monoclonal Antibodies 3A.1-6 and BK**

Keratinocytes were seeded onto 12-mm circular glass chips placed in 35-sq mm dishes and were allowed to grow in complete MCDB 153 for 1–2 days. Cells were then growth arrested and induced to differentiate by the methods described. Specimens were thereafter fixed for 8 minutes with 3.7% formaldehyde in phosphate-buffered saline (PBS), then with methanol for 4 minutes (−20 C), and finally with acetone for 2 minutes (−20 C). They were then air-dried. The glass chips were subsequently incubated for 30–60 minutes in a humidified chamber at room temperature with the antibody 3A.1-6, which was prepared by Drs. Gregg Hadley and David Steinmuller, University of Michigan, and which is a keratinocyte differentiation marker that stains high suprabasal cells in human skin,<sup>7</sup> or the antibody 53.A1 (herein designated “BK”), which was obtained as a gift from Dr. James Clagett, University of Washington, and which selectively stains more undifferentiated basal cells in human skin (unpublished information). The antibodies 3A.1-6 and BK were used at dilutions of 1:1000 and 1:30, respectively. After incubation with the primary antibody, the specimens were washed in PBS and incubated for an additional 30–60 minutes with fluorescein-conjugated rabbit antimouse immunoglobulin (Cappel Laboratories, Cochranville, Pa) at a 1:15 dilution at room temperature in a humidified chamber. Finally, the specimens were washed in PBS, mounted, and examined with a Zeiss fluorescence mi-

croscope as described above. All immunologic assays employed a variety of controls. For example, negative controls included specimens that were not incubated with the primary antibody or that were incubated instead with various preimmune sera.

## **Results**

The standard procedure that we have used in our previous studies to induce terminal differentiation in rapidly growing human keratinocytes is to culture cells in serum-free medium MCDB 153 in which the growth factors including insulin, EGF, and BPE have been removed and the calcium concentration of the medium has been increased to 2.0 mM.<sup>2</sup> Under these culture conditions the cells organize into colonies that have a complex structure wherein the periphery of the colony contains cells that retain proliferative potential, and the cells in the center of the colony irreversibly growth arrest, stratify, and differentiate.<sup>2</sup> Although this is an excellent method of inducing terminal differentiation, with this method it is difficult to evaluate the sequence of events that occurs during terminal differentiation, because the differentiated colonies contain heterogenic cell populations with respect to proliferation and differentiation characteristics.

In order to study in more detail the role of growth arrest at either a reversible and/or irreversible state with respect to the control of differentiation, we performed the current studies using more homogeneous cell populations that had been growth-arrested at distinct types of states and then incubated in differentiation-promoting medium.

#### **Methods of Growth Arrest**

Reversible arrest of keratinocyte growth has been reported to be induced by medium containing TGF- $\beta$  (5 ng/ml) and isoleucine-deficient medium.<sup>3</sup> Cells cultured in these media show markedly decreased <sup>3</sup>H-thymidine incorporation into DNA and the preferential inhibition of cell cycle progression in G<sub>1</sub>. Most important, clonogenic assays established that when initially growth-arrested, these keratinocyte populations could be restimulated to proliferate when passaged and refed complete MCDB 153; thereafter, colony-forming efficiency was greater than 80% of the control. We now report that culture of cells in complete MCDB 153 and 8 mM ethionine for 2 days initially induces a type of reversible growth arrest similar to that documented in Table 1. That is, when cells are incubated in ethionine-containing medium, they initially growth-arrest predominantly in G<sub>1</sub> in a reversible state, wherein they retain a high clonogenic

Table 1—Characterization of Reversible and Irreversible Types of Growth Arrest States in Normal Human Keratinocytes\*

	Characterization of growth arrest by <sup>3</sup> H-thymidine incorporation into DNA (% LN)†	Cell cycle distribution (%)			Clonogenic potential (% CFE)‡
		G <sub>1</sub>	S	G <sub>2</sub> /M	
Controls: rapid growth state	95	48	35	17	100
Reversible growth arrest state					
TGF- $\beta$ containing medium	3	75	5	20	90
Isoleucine-deficient medium	<1	80	3	17	80
Ethionine containing medium	1	76	10	14	90
Irreversible growth arrest state					
Razoxane containing medium	5	5	5	90	<5
Senescence in complete medium	<1	92	3	5	0

\* Some of the data in this table summarize the results of previous studies that form the basis for the current experiments (see Pittelkow et al<sup>3</sup>).

† Percent labeled nuclei.

‡ Percent colony-forming efficiency (CFE) is expressed as the CFE of an experimental culture relative to that of rapidly growing cells which are designated to be 100%.

potential. (Subsequently, the stability of these reversible growth arrest states will be considered in detail.)

Irreversible growth arrest of human keratinocytes has also been reported after their culture in razoxane-containing medium.<sup>3</sup> In addition, we have shown that keratinocytes cultured in complete MCDB 153 containing 0.1 mM calcium undergo senescence,<sup>2</sup> which by definition is also an irreversible growth arrest state, and we now show that they can do so without differentiating. A review of the data on the irreversible growth arrest states is also presented in Table 1.

Cells at the above growth arrest states represent relatively homogeneous populations of undifferentiated keratinocytes. We therefore next evaluated whether differentiation could be induced from one or more of these states.

### Control of Differentiation From Irreversible Growth Arrest States

Because in most experimental systems differentiation of keratinocytes is associated with irreversible loss of growth potential, we initially evaluated whether undifferentiated keratinocytes that were irreversibly growth arrested by senescence or by culture

in medium containing razoxane could be induced to differentiate. To do this, we prepared cells at these two irreversible growth arrest states, then refed them growth factor-deficient MCDB 153 medium containing 2 mM Ca<sup>2+</sup> and then assayed differentiation over a 6-day interval. Table 2 lists the differentiation assays that were used and justifies their use with appropriate references.

The results show that differentiation can be induced in irreversibly arrested razoxane-treated and senescent keratinocytes. Table 3 summarizes the data and documents that after culture of such cells in medium containing 2 mM calcium, 50–90% of the cells were induced to differentiate. Differentiation was demonstrated by morphologic evidence of stratification and crown formation and histochemical staining of cells by the Ayoub and Shklar procedure. Figure 1 illustrates a typical example of the morphology of undifferentiated keratinocytes relative to differentiated keratinocytes that were induced to stratify by culture in growth factor-deficient medium containing 2 mM Ca<sup>2+</sup> medium. Differentiation was also evidenced by the presence of involucrin in approximately 50% of such cells. In addition, differentiated colonies showed a marked loss of the basal keratinocyte antigen de-

Table 2—Assays to Quantitate the Extent of Keratinocyte Differentiation

Assay	Nondifferentiated cultures	Differentiated cultures	Reference
I. Ayoub and Shklar red histochemical staining	—	+	5, 6
II. Stratification and crown formation	—	+	8, 9
III. Immunofluorescent studies			
a. Involucrin polyclonal antibody	—	+	10
b. BK monoclonal antibody	+	—	*
c. 3A.1-6 monoclonal antibody	—	+	7

\* Unpublished data.

Table 3—Differentiation Potential of Irreversibly Growth-Arrested Human Keratinocytes

	Differentiation assays		
	% Colonies expressing stratified crowns*	% Colonies expressing differentiated foci by the Ayoub and Shklar histochemical procedure†	% Involucrin-positive cells‡
I. Differentiation characteristics of cells in native growth arrest-inducing medium			
Razoxane arrest state	0	0	3
Senescence arrest state	0	0	3
II. Differentiation characteristics of cells after incubation in differentiation-promoting medium§			
Razoxane arrest state	92	79	48
Senescence arrest state	≥80	≥80	60

\* Percent crown formation was determined as the percent of colonies displaying multilayered stratification as determined by phase-contrast microscopy.

† A colony was scored as differentiated if it contained a red foci of cells after cytochemical staining procedures of Ayoub and Shklar.

‡ Percent cells expressing involucrin was determined from indirect immunofluorescent antibody studies.

§ Cultures were growth-arrested as described, then refed and cultured for 6 days in native growth-arresting medium that contained 2 mM  $\text{Ca}^{2+}$  but which was devoid of growth factors.

tected by the BK monoclonal antibody and the appearance of a keratinocyte differentiation antigen to the 3A.1-6 monoclonal antibody (data not presented).

### Control of Differentiation From Reversible Growth Arrest States

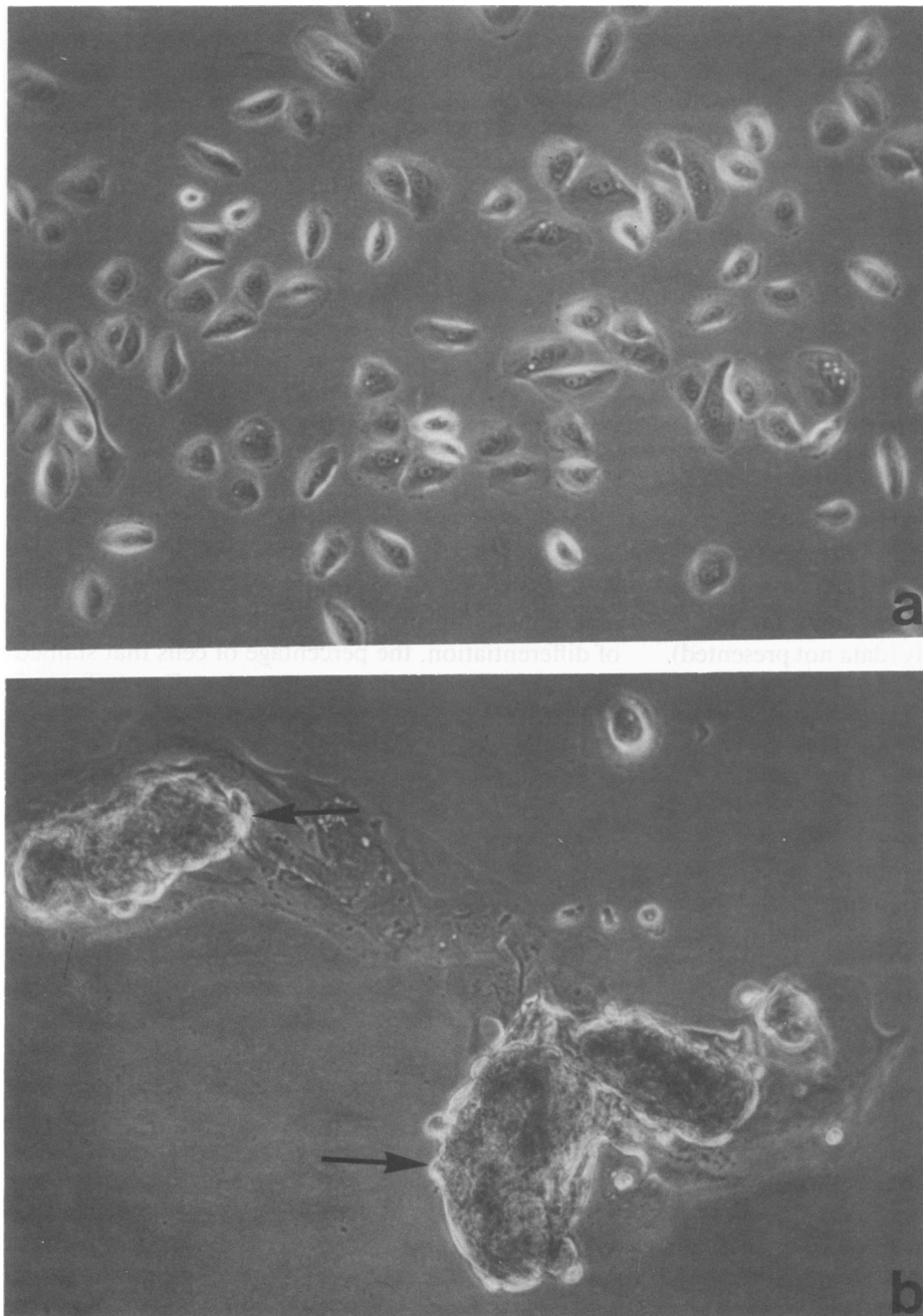
Because human keratinocytes can also be growth-arrested at reversible states, we next asked whether such cells could also be induced to differentiate. Table 4 summarizes the data and shows that keratinocytes that initially undergo reversible growth arrest in TGF- $\beta$ , isoleucine deficiency, or ethionine show no significant evidence of differentiation. However, 6 days after refeeding such cultures differentiation-inducing medium, the cells showed significant stratification and differentiation, as detected by use of the Ayoub and Shklar stain. In addition, a significant population of cells stained positive for involucrin. In general, 30–70% of such cells expressed differentiated characteristics under these culture conditions.

Having shown that differentiation can be induced from cells initially growth-arrested at a reversible state, a series of experiments was next performed to assure that the reversibly arrested cells did not undergo cell cycle progression after addition of the differentiation-promoting medium and prior to terminal differentiation. That is, experiments were performed to assure that arrested cells can terminally differentiate without passing through the S phase of the cell cycle wherein DNA synthesis occurs. Autoradiographic analysis of  $^3\text{H}$ -thymidine incorporation into DNA was therefore evaluated over the 6-day interval during which differentiation occurred in

growth-arrested cell populations that were refed differentiation-promoting medium. For quantitation of differentiation, the percentage of cells that stained positive for involucrin was assayed on Day 6. Table 5 summarizes the data and shows that at least 23% of TGF- $\beta$ -arrested, 28% of isoleucine-arrested, and 52% of ethionine-arrested cells are capable of differentiation without entering DNA synthesis. Because of the technical limitations of such experiments, these data represent the minimum percentage of cells that can differentiate without DNA synthesis.

### Role of Growth Factors and Calcium in the Induction of Differentiation

In most of the above studies growth factor-deficient medium containing 2 mM  $\text{Ca}^{2+}$  was used for inducing differentiation; we therefore next evaluated the relative importance of growth factor deficiency versus 2 mM calcium in inducing differentiation. More specifically, keratinocytes that were growth-arrested in ethionine-containing medium, razoxane-containing medium, or isoleucine-deficient medium were cultured in either growth factor-deficient MCDB 153 containing 2 mM  $\text{Ca}^{2+}$  or in complete MCDB 153 containing 2 mM calcium. Thereafter, differentiation assays were sequentially performed. Table 6 summarizes the results from ethionine-arrested cells and shows that growth factor-complete medium containing 2 mM calcium is comparable to growth factor-deficient medium containing 2 mM  $\text{Ca}^{2+}$  in inducing differentiation. Similar data were obtained with cells at the other two arrest states. Therefore, growth factor deficiency is not the primary differentiation-inducing agent for these growth-arrested cells.



**Figure 1**—Typical morphology of growth-arrested human keratinocytes before (a) and after (b) induction of differentiation in medium containing 2 mM Ca<sup>2+</sup>. The differentiated cultures show aggregates of cells that form colonies and stratify and express differentiation-specific antigens such as involucrin. (Phase micrographs,  $\times 94$ )

### Stability of Reversible Growth Arrest

Reversible growth arrest of human keratinocytes has been shown to be induced by culture in TGF- $\beta$ , ethionine, or isoleucine-deficient media for 48–72 hours. To determine whether more prolonged culture in such medium maintains growth arrest at a reversible state or whether progression to an irreversible state occurs, we performed another series of experiments. More specifically, cells were evaluated for their

clonogenic potential after maintenance in TGF- $\beta$  or ethionine for up to 12 days. (The use of isoleucine-deficient medium was not employed because the deficiency of isoleucine in the culture medium for such prolonged intervals resulted in significant toxicity.) These studies were performed in complete MCDB 153 medium containing a low calcium concentration, ie, 0.1 mM.

The results presented in Table 7 show that a significant percentage of cells cultured in medium con-

Table 4—Differentiation Potential of Human Keratinocytes Initially Growth-Arrested at a Reversible State

	Differentiation assays		
	% Colonies expressing stratified crowns	% Colonies expressing differentiated foci by the Ayoub and Shklar histochemical procedure	% Involucrin-positive cells
I. Differentiation characteristics of cells in native growth arrest-inducing medium			
Ethionine arrest state	0	0	0
Isoleucine-deficient arrest state	0	0	2
TGF- $\beta$ arrest state	0	ND	1
II. Differentiation characteristics of cells after incubation in differentiation-promoting medium			
Ethionine arrest state	70	68	52
Isoleucine-deficient arrest state	38	35	29
TGF- $\beta$ arrest state	61	ND	37

See legend to Table 3.

ND, not determined.

taining TGF- $\beta$  remained growth-arrested at a reversible restriction point even after 12 days in culture and that they showed no evidence of significant differentiation as evaluated by the expression of involucrin. In contrast, cells maintained in ethionine-containing medium gradually progressed to an irreversible arrest state and differentiated even in low-calcium-containing medium, ie, 0.1 mM.

These observations suggest that the reversible arrest states induced by TGF- $\beta$  and ethionine are distinct, that the process of keratinocyte differentiation involves a series of biologic steps, and that at certain states differentiation can occur quite well even in the absence of 2 mM  $\text{Ca}^{2+}$ . To further dissect the steps involved in the differentiation process, additional kinetic studies were performed for determining how many steps are involved in the process of normal human keratinocyte differentiation.

### Steps in the Process of Terminal Differentiation

Based on the data presented previously in this paper, our working proposal was that cells first reversibly growth-arrest, then irreversibly lose proliferative

potential and subsequently express overt keratinocyte differentiation. For a more direct test of this possibility, cells were reversibly growth arrested in ethionine containing complete MCDB 153 medium. They were then maintained in the same medium, refed complete MCDB 153 + ethionine + 2 mM calcium, or refed growth factor-deficient MCDB 153 medium containing ethionine + 2 mM calcium. Thereafter, the proliferative potential of the cells was determined by clonogenic assays, and the differentiation potential of the cells was determined by evaluation of crown formation and involucrin expression. Figure 2 documents the results of two of these studies and shows that between Day 3 and Day 5, when the cells become reversibly arrested, they maintain a high colony-forming efficiency and are not differentiated. Thereafter, especially between Day 5 and Day 7 the cells progress to an irreversible arrest state wherein they show markedly reduced clonogenic potential but still do not differentiate. Finally, after 9–11 days the irreversibly arrested cells express overt differentiation. These data confirm our hypothesis that there are at least three steps involved in terminal

Table 5—Quantitation of the Percent Keratinocytes Capable of Differentiating Without Entering DNA Synthesis From Reversible Growth Arrest States

Inducer of reversible growth arrest prior to addition of differentiation-promoting medium	Predominant cell cycle state	% Labeled nuclei during differentiation*	% Involucrin positive cells†	% Cells that can differentiate without DNA synthesis
Ethionine	G <sub>1</sub>	0	52	>52
Isoleucine-deficient	G <sub>1</sub>	1	29	>28
TGF- $\beta$	G <sub>1</sub>	14	37	>23

\* Percent labeled nuclei after induction of differentiation represent the cumulative labeling of nuclei during consecutive 48-hour labeling intervals for the 6-day differentiation-inducing period.

† Percent cells positive for involucrin was determined from indirect immunofluorescent antibody studies.

Table 6—Relative Effect of Growth Factor-Deficient 2 mM  $\text{Ca}^{2+}$  Medium Relative to Growth Factor-Complete 2 mM  $\text{Ca}^{2+}$  Medium on the Differentiation of Ethionine-Arrested Keratinocytes

	Differentiation assays		
	% Colonies expressing stratified crowns	% Colonies expressing differentiated foci by the Ayoub and Shklar histochemical procedure	% Involucrin-positive cells
Growth factor-deficient medium + 2 mM $\text{Ca}^{2+}$	100	100	41
Growth factor complete medium + 2 mM $\text{Ca}^{2+}$	69	94	38

See legend to Table 3.

keratinocyte differentiation: 1) reversible growth arrest, 2) irreversible loss of proliferative potential, and 3) the expression of the terminally differentiated phenotype. A model to illustrate this conclusion is presented in Figure 3.

### Discussion

The regulation of proliferation in many cell types is physiologically associated with cell differentiation. Previous studies on murine mesenchymal stem cells<sup>11-15</sup> and on myogenic<sup>16</sup> and teratocarcinoma cell lines<sup>17</sup> have in fact established that a specific relationship exists between growth arrest and differentiation. Our studies have also established that the control of normal human keratinocyte proliferation and differentiation is integrally regulated<sup>2</sup>; however, the precise relationship between growth arrest and differentiation in normal human keratinocytes has not been clearly documented.

Using normal human keratinocytes cultured in serum-free medium, we now report that differentiation occurs after growth arrest at either of two general types of biologic state. The results show that differentiation can be induced in keratinocytes that are irreversibly growth-arrested by culture in razoxane-containing medium or by senescence. Differentiation can

also be induced in keratinocytes that are initially growth-arrested at a reversible state by culture in medium containing either TGF- $\beta$  or ethionine or in medium deficient in isoleucine. The effect of these factors on keratinocyte proliferation and differentiation and the role of calcium in this process are somewhat complex because the data presented in this paper demonstrate that three steps are involved in keratinocyte differentiation. Cells first undergo reversible pre-differentiation growth arrest, and subsequently such cells become irreversibly growth-arrested and lose their proliferative potential. Only thereafter is the terminally differentiated phenotype expressed. Figure 3 presents a model that summarizes these observations, and Figure 4 illustrates more specifically the precise effect of various factors and calcium concentrations on normal human keratinocyte growth arrest and differentiation.

These new observations raise a series of important questions, including some that cannot currently be answered and some that can. One important, yet unanswered question concerns whether these results obtained on cultured human keratinocytes reflect the characteristics of keratinocytes *in vivo*. It would certainly be worthwhile to determine whether there are physiologic growth arrest states in keratinocytes *in vivo* from which they make decisions either to proliferate

Table 7—Stability of the Reversible Growth Arrest States Induced by TGF- $\beta$  and Ethionine

Growth arrest culture condition	Duration of culture (days)	Viability (% controls)*	Colony-forming efficiency (% controls)*	Differentiation (% maximum involucrin positive cells)†
I. Complete MCDB 153 (0.1 mM $\text{Ca}^{2+}$ ) + TGF- $\beta$	3	81	89	—
	6	97	43	—
	9	100	36	—
	12	78	37	$\leq 1$
II. Complete MCDB 153 (0.1 mM $\text{Ca}^{2+}$ ) + ethionine	3	93	100	—
	6	100	41	—
	9	100	26	—
	12	70	3	62

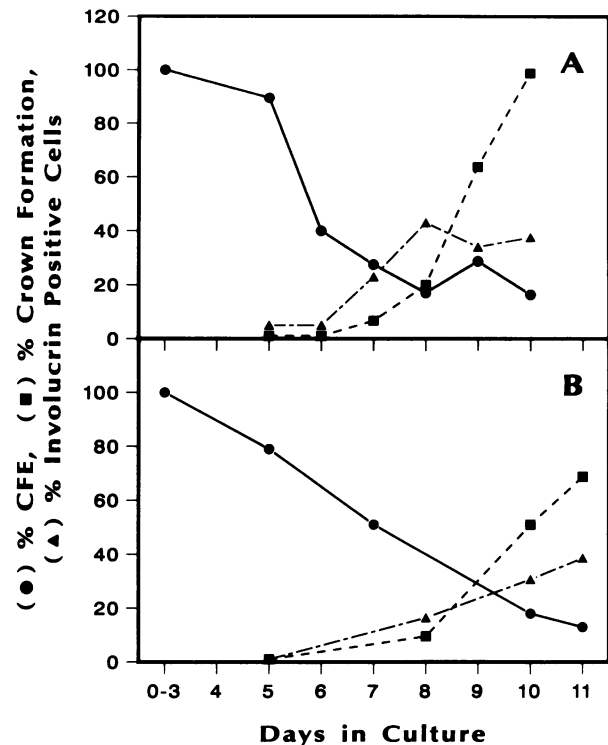
\* Controls for these studies were rapidly growing cells.

† The maximum extent of involucrin expression of approximately 35–50% is observed when keratinocytes are cultured in growth factor-deficient MCDB 153 + 2 mM  $\text{Ca}^{2+}$ .

erate or differentiate in association with the irreversible loss of proliferative potential. That question cannot be specifically answered because it is extremely difficult to obtain unambiguous *in vivo* experimental evidence concerning whether subpopulations of cells exist at various growth arrest states prior to their differentiation.<sup>18</sup>

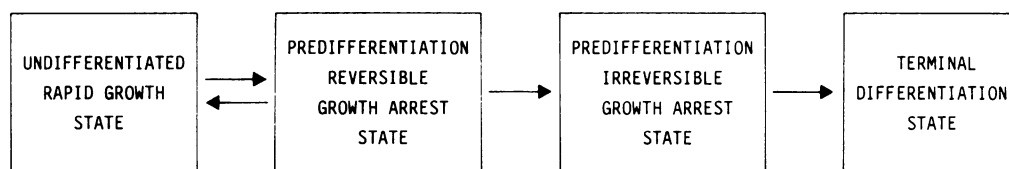
A question that can, however, be answered concerns how the current data on cultured keratinocytes correlate with our previous data on murine mesenchymal stem (3T3 T) cells, wherein we established their mechanisms to regulate integrally the control of proliferation and differentiation.<sup>11-15</sup> In both keratinocytes and 3T3 T cells, differentiation specifically occurs after growth arrest at a reversible predifferentiation state. In both cell types, terminal differentiation is a multistep process, and in both cell types the transition from the predifferentiation growth arrest state to the terminal differentiation state is associated with a loss of growth potential. There are, however, also dissimilarities between 3T3 T cells and keratinocytes. For example, the regulation of differentiation in 3T3 T cells is mediated at a distinct growth arrest state in the G<sub>1</sub> phase of the cell cycle; whereas in keratinocytes, differentiation can be induced from several different cell cycle states in either G<sub>1</sub> or G<sub>2</sub>. Furthermore, in the 3T3 T cells overt differentiation can occur in cells that retain proliferative potential, while in keratinocytes proliferative potential appears to be lost prior to overt differentiation.

These differences between 3T3 T cells and keratinocytes could have a variety of logical reasons. 3T3 T cells are of mouse origin, whereas keratinocytes are human. 3T3 T cells are of mesenchymal origin, whereas keratinocytes are epithelial. 3T3 T cells are also immortal and aneuploid, whereas keratinocytes are mortal and diploid. Notwithstanding these possible explanations, we think that the most interesting explanation for these differences is the fact that 3T3 T cells are stem cells, whereas keratinocytes are progenitor cells. In this regard, we suggest that stem cells must possess a nearly unlimited proliferation potential and multiple differentiation potentialities,<sup>19,20</sup> whereas progenitor cells have a markedly limited proliferation

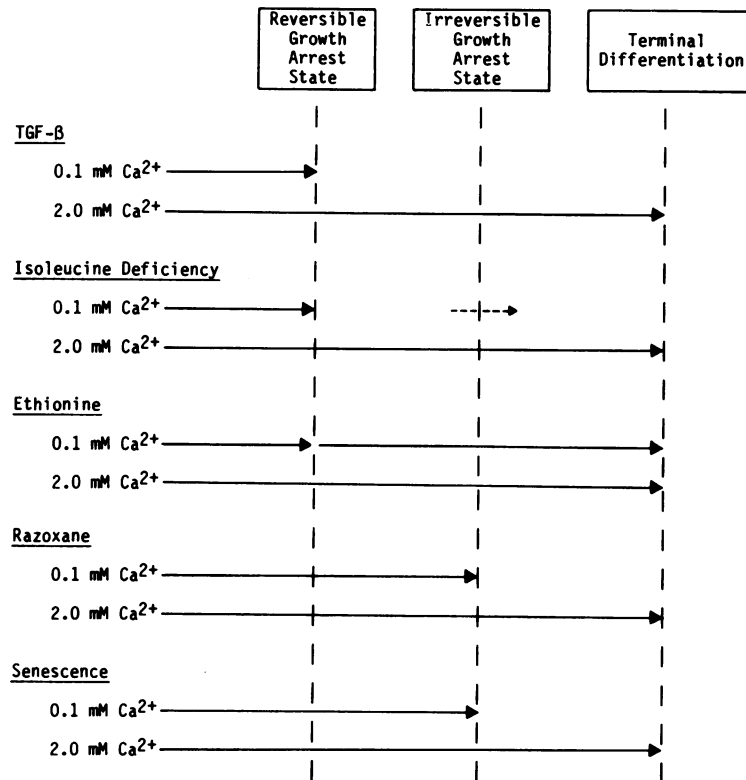


**Figure 2**—Proliferation and differentiation potential of ethionine growth-arrested keratinocytes induced to differentiate. Cultures were plated at approximately  $2 \times 10^3$  cells/sq cm at Day 0 and allowed to grow for 3 days, at which time they were refed ethionine-containing complete MCDB 153 medium (0.1 mM Ca<sup>2+</sup>). After 2 days in ethionine-containing medium, when reversible growth arrest had occurred, cultures were induced to differentiate either by refeding growth factor-deficient MCDB 153 medium containing 2 mM Ca<sup>2+</sup> plus ethionine (a) or by refeding growth factor-complete MCDB 153 medium containing 2 mM Ca<sup>2+</sup> plus ethionine (b). Colony-forming efficiency (CFE) (●—●) was expressed as percent of rapidly growing control cultures; the percent of colonies expressing crown formation (■---■) and the percent of involucrin positive cells (▲---▲) were then sequentially assayed.

and differentiation potential. Evidence that 3T3 T cells represent multipotential mesenchymal stem cells has been well documented,<sup>14,15</sup> and more recent data support the conclusion that keratinocytes are progenitor cells. First, the keratinocytes that grow in culture do not express unrestricted proliferative potential and in fact become senescent after 30–50 population doubling.<sup>2</sup> Cultured keratinocytes are also derived from partially differentiated suprabasal cells in the skin, rather than from less differentiated basal cells. Finally,



**Figure 3**—Model for the steps involved in the integrated control of proliferation and differentiation in cultured normal human keratinocytes.



**Figure 4**—Summary of the effect of different pharmacologic and physiologic factors on normal human keratinocyte proliferation and differentiation. The figure illustrates that TGF- $\beta$ /0.1 mM  $\text{Ca}^{2+}$  induces stable reversible growth arrest and that increasing the calcium concentration to 2.0 mM in the presence of TGF- $\beta$  induces reversibly arrested cells to proceed to a terminally differentiated state. A similar effect is observed for cells arrested by isoleucine deficiency, although in such cells it is not possible to determine how stable the reversible arrest state is in low-calcium-containing medium because of toxicity, as indicated by the dotted arrow. The data on ethionine-arrested cultures is, however, distinct. The cells in ethionine initially growth-arrest in a reversible state, but subsequently they slowly progress to an irreversible growth arrest state and finally to a terminally differentiated state even in medium containing 0.1 mM  $\text{Ca}^{2+}$ . A high calcium concentration appears only to facilitate this process in such cells. Finally, the figure shows that both razoxane-treated and senescent cells irreversibly arrest growth at an undifferentiated state in medium containing 0.1 mM  $\text{Ca}^{2+}$  but that subsequently such cells can be induced to differentiate terminally when the calcium concentration is raised to 2 mM.

cultured keratinocytes can only differentiate into a single cell type *in vitro*; when such cultured keratinocytes are grafted onto the dermis of burn patients, they only differentiate into keratinocytes and do not give rise to other epithelial cell types, such as epithelial cells that form sweat glands, sebaceous glands, or hair follicles.<sup>21</sup>

In summary, in this paper we have established for the first time the complex biologic processes that integrally regulate the proliferation and differentiation of normal human keratinocyte progenitor cells. We now suggest that future studies need to be performed to establish the molecular mechanisms that control proliferation and differentiation in cultured keratinocytes and to determine whether significant differences exist in the regulatory mechanisms in human squamous carcinoma cells. Additional studies to develop methods to culture true epithelial stem cells are also most important because such stem cells are almost certainly the source of the  $\geq 80\%$  of all human cancers, ie, epithelial-derived carcinomas.

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